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"Treatment and prevention of breast cancer using multifunctional inhibitors of cholesterol biosynthesis"

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Note: An abstract is required to be provided in Block 14

Most human breast cancers are hormone responsive, depending on estrogens and progestins for tumor cell proliferation. Initially, hormone-responsive tumors respond to endocrine therapy, however, most human breast tumors develop resistance to currently used endocrine therapeutic protocols. It is therefore essential that we identify additional molecular targets in the signaling pathways that lead to tumor growth if we are to effectively treat and prevent cancers of the breast. It is well-established that breast cancer cells have the capacity to synthesize endogenous cholesterol, the precursor for steroid hormones. Cholesterol biosynthesis by tumor cells therefore potentially contributes towards anti-hormone resistance. Most commonly used cholesterol lowering drugs inhibit HMG CoA-reductase, a key rate-limiting enzyme in the cholesterol biosynthetic pathway; these inhibitors are however associated with certain undesirable side effects that limit their use for cancer therapy. Our goal was to identify alternative targets in the pathway leading to the production of cholesterol, which might be regulated with less toxic inhibitors to control the progression of breast disease. Inhibitors of oxidosqualene cyclase (OSC), an enzyme down-stream of HMG CoA-reductase, effectively arrested breast cancer cell proliferation. RO0488071 [4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]) (RO), and an analogue RO0613479, were particularly effective in this regard. Administration of both of these OSC inhibitors to ER positive human breast cancer cells (e.g. BT-474, T47-D, MCF-7 cells) at a pharmacological dose or at a dose close to the IC_{50} value for OSC (nM range) reduced tumor cell viability in vitro. Initial studies show that administration of RO to animals with human breast cancer cell-derived xenografts prevents further in vivo progression of the disease, with no apparent toxicity. Since BT-474 cells are also tamoxifen resistant and rich in Her2/neu, RO appears to be effective in vivo against even the most aggressive anti-hormone resistant tumors. Importantly, RO had no effect on the viability of normal human mammary cells. Our study shows for the first time that inhibition of cholesterol biosynthesis using OSC inhibitors is a novel and potent means by which to destroy human breast cancer cells, though further studies are necessary to determine the mechanism of RO mediated loss of breast cancer cell viability.

Table of Contents

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The purpose of this research is to investigate whether a cholesterol synthesis inhibitor is also an effective therapeutic drug which can be used to control the progression of breast cancer. The effects of the drug on a number of different breast cancer cell lines will be examined, as well as its in vivo effect against breast cancer cells grown in xenograft models, using both mono- and combination therapy protocols.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Breast Cancer, Cholesterol inhibitors, therapeutics, Cell viability, apoptosis

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Progress related to Task 1. ***Characterize the impact of RO on estrogen signaling in breast cancer cells. (estrogen receptor alpha=ERα; estrogen receptor beta=ERβ)***

Goals:

a. Determine the effect of RO therapy on cell viability using a number of different breast cancer cells, and normal mammary cells.
b. Determine the level of ERα and ERβ expression in treated cells used by Western blot analysis.
c. Determine the effect of RO treatment on estrogen-dependent proliferation of breast cancer cells.
d. Determine whether RO treated ERα positive cells lose their capacity to regulate ERα-dependent gene regulation but retain regulation of ERβ specific genes with ERβ-interacting ligands.
e. Determine whether RO influences transcription of ERα and ERβ genes in breast cancer cells.
f. Determine whether RO influences stability of ERα and ERβ protein in breast cancer cells.

Substantial progress has been made with respect to Task 1. We have shown that the cholesterol lowering drug RO and its analogues suppress the viability of a number of different breast cancer

cell lines in a dose-dependent manner, without affecting normal mammary cells (Fig 1 A-D). RO appears to be a more potent inhibitor of breast cancer cells than statins, which are generally used to lower cholesterol (Fig 1E). Furthermore, using Western blot analysis we demonstrate that RO degrades ERα in various breast cancer cells in a time and dose dependent manner (Fig 2A). ERα degradation is dependent on proteosome mediated protein loss (Fig 2B). Importantly, simultaneously with ERα degradation, ERβ, an anti-proliferative protein in breast cancer cells is also induced (Fig2C). This induction of ERβ and loss of ERα also occurs with low concentrations of RO when the inhibitor is administered for a longer time (Fig 2D). Based on our real-time PCR analysis data it appears that induction of ERβ is independent of transcriptional events (not shown) and may involve RO mediated receptor stabilization, a scenario we are investigating further. RO also suppresses estrogen-dependent cell proliferation, presumably as a consequence of inducing ERβ, while reducing ERα (Fig 3). We are now conducting studies to determine whether ER-dependent gene expression is influenced by RO-mediated events in modulating levels of ERs. We are also determining whether RO influences protein stability of ERs in tumor cells.

Progress related to Task 2. ***Characterize the in vitro effects of RO mono- or combination therapy on proliferation and apoptosis of breast cancer cells in vitro.***

Goals:

a. Measure apoptosis in breast cancer cells treated with either RO alone or in combination with ERβ interacting ligands.
b. Determine protein levels of apoptosis related genes (p21, caspase-3, Bcl-2, Bad, Bax) following treatment of cells with RO.
c. Initiate combination therapy, keeping the concentration of one ligand constant while varying that of the other to determine whether there are additive or synergistic effects on apoptosis.
d. Determine mRNA and protein levels of proteins related to apoptosis and angiogenesis, such as p21, caspase-3, Bcl-2, Bad, Bax, and VEGF following treatment of cells with RO in combination with aforementioned compounds.
e. Transfect cells with siRNA to down-regulate ERβ and determine cell viability and response to RO using cell-proliferation assays.
f. Following ERβ and OSC siRNA transfections, test breast cancer cells for lack of sensitivity to RO in order to define a molecular target for mediating RO effects. These experiments will utilize cell viability assays.

A number of studies described in task 2 have been completed and many are on-going. We have shown that RO induces apoptosis in a number of different breast cancer cell lines when administered either alone or in combination with an ERβ antagonist, using both FACS (Fig 4 A-B) and SRB assays (Fig 4C). Further studies are underway using additional ERβ interacting ligands. In a limited set of studies we have identified induction of p21 and loss of Bcl-2, confirming that RO destroys tumor cells (Fig 5 A-B). After transfecting a limited number of cells with siRNA against ERβ and administering RO we observed a diminished effect on the promotion of apoptosis, suggesting that ERβ is one of the targets for RO mediated anti-cancer effects (Fig 6). We need to perform several of these studies using multiple cell lines.

Progress related to Task 3. ***Characterize the effects of RO mono- or combination therapy on progression and prevention of breast cancer cells in vivo in rodent models.***

Goals:

a. Breast cancer cells in matrigel will be inoculated into nude mice (6-8 week-old, female, nu/nu, sc).

b. Tumors will be allowed to reach 100-200mm^3 in size, after which animals will be randomly assigned to groups for treatment with RO or vehicle alone. Treatment with RO (5-25 mg/kg, iv) will be once a day for 10 days.

c. Experiment in b) will be repeated in vivo using a combination of RO and an ERβ specific ligand, as well as RO and a natural compound which has an affinity for ERβ, to determine additive or synergistic effects in reducing in vivo tumor progression.

d. Tumor samples will be collected after the first three injections and then again at the end of the experiment in b. and c. for further analysis by immunohistochemistry. Samples will also be saved in liquid nitrogen for Western blot analysis and RNA isolation.

e. Western Blot and RT-PCR will be used to analyze protein levels and RNA expression of ER, PR , p21, Caspase-3 and VEGF.

f. Immunohistochemistry will be used to quantitate blood vessel density and various antigens indicated in e.

In vivo studies using RO are underway. We have already shown that RO effectively controls the progression of estrogen and progesterone receptor positive breast cancer in animals, without toxicity (Fig 7A-B). We are currently awaiting results from in vitro combination therapy and will utilize those ERβ interacting ligands which prove most effective in vitro, in our in vivo studies. These experiments will be the focus of our studies in year 2 of the proposal, in conjunction with immunohistochemical analysis of tumor tissue to determine the mechanism of action of RO. Other studies in tasks 1 and 2 will also be finalized in year 2.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

    - RO blocks proliferation of breast cancer cells
    - RO also blocks estrogen-induced proliferation of breast cancer cells
    - RO appears to be a better antagonist for blocking proliferation of breast cancer cells than the commonly used statins
    - RO induces apoptosis of tumor cells, which explains how it controls the growth of breast cancer cells
    - RO induces p21 and reduces Bcl2 expression, which at least partially explains its mechanistic effects with respect to controlling tumor cell progression

- In initial studies, it appears that RO is an effective drug when used in a monotherapy mode to reduce the progression of breast tumors in vivo

5. CONCLUSION: Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

Our research, with the support of this grant, shows that cholesterol inhibitors that target OSC induce tumor cell apoptosis and can therefore be used to prevent the progression of breast cancer cells. In addition OSC inhibitors have off-target effects; they degrade estrogen receptor alpha, a major pro-proliferative protein in hormone-responsive cells, and induce estrogen receptor beta protein, a major factor which reduces cell proliferation. Once the studies proposed in the grant are complete, they will yield information vital to determining whether the use of these drugs is feasible in humans. If so one can move towards human clinical trials which, we believe, could set the stage for the therapeutic use of OSC inhibitors against breast cancer and potentially save millions of lives worldwide.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:
Nothing to report

(2) Peer-Reviewed Scientific Journals:
Manuscript is in preparation

(3) Invited Articles:
Hyder, S. M., Mafuvadze, B and Besch-Williford, C. (2013) Novel Anti-Angiogenic Therapies using Naturally-Occuring and Synthetic Drugs to Combat Progestin-Dependent Breast Cancer to be published in Cell and Molecular Biology of Breast Cancer, Humana Press, In Press

(4) Abstracts:

Liang, Y., Zou, X., Besch-Williford, C., Johnnes, A. and Hyder , S. M. (2013) Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. 103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871 (as stated above)

7. INVENTIONS, PATENTS AND LICENSES: List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report

8. REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

All the results described in Section 3 are reportable and will soon be in the form of a manuscript. The results show a substantial advance towards a potentially new therapeutic protocol for breast cancer which could involve the use of specific cholesterol lowering drugs that target oxidosqualene cyclase. These drugs may be administered with or without additional drugs that target the estrogen signaling mechanisms. Evidence for such a possibility comes from our observation that estrogen receptor beta specific ligands appear to enhance the effects of cholesterol lowering drugs. It is also possible that such an approach could also prove useful for actually preventing breast cancer.

9. OTHER ACHIEVEMENTS: This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Nothing to report

For each section, 4 through 9, if there is no reportable outcome, state “Nothing to report.”

10. **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

    n/a

11. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

    Please see attached

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under "Forms" on https://www.usamraa.army.mil) should be updated and submitted with attachments.

MARKING OF PROPRIETARY INFORMATION: Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE. It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS. See term entitled "Intangible Property – Data and Software Requirements" and https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.

Presented at the 103rd Annual American Association of Cancer Research Meeting (2013), Washington DC, USA, Abstract #871

Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. Yayun Liang[1,2], Xiaoqin Zou[1,3] , Cynthia Besch-Williford[4], Johannes Aebi[5] and Salman M Hyder[1,2]. Dalton Cardiovascular Research Center[1] & Dept of Biomedical Sciences[2], Department of Physics and Astronomy, Department of Biochemistry, and Informatics Institute[3], University of Missouri, Columbia, MO 65211, IDDEX RADIL[4], Columbia, MO, 65201, and F. Hoffmann-La Roche Ltd., Pharmaceutical Division, CH-4070 Basel, Switzerland[5].

Most human breast cancers are hormone responsive, depending on estrogens and progestins for tumor cell proliferation. Initially, hormone-responsive tumors respond to endocrine therapy, however, most human breast tumors develop resistance to currently used endocrine therapeutic protocols. It is therefore essential that we identify additional molecular targets in the signaling pathways that lead to tumor growth if we are to effectively treat and prevent cancers of the breast. It is well-established that breast cancer cells have the capacity to synthesize endogenous cholesterol, the precursor for steroid hormones. Cholesterol biosynthesis by tumor cells therefore potentially contributes towards anti-hormone resistance. Most commonly used cholesterol lowering drugs inhibit HMG CoA-reductase, a key rate-limiting enzyme in the cholesterol biosynthetic pathway; these inhibitors are however associated with certain undesirable side effects that limit their use for cancer therapy. Our goal was to identify alternative targets in the pathway leading to the production of cholesterol, which might be regulated with less toxic inhibitors to control the progression of breast disease. Inhibitors of oxidosqualene cyclase (OSC), an enzyme down-stream of HMG CoA-reductase, effectively arrested breast cancer cell proliferation. RO0488071 [4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]) (RO), and an analogue RO0613479, were particularly effective in this regard. Administration of both of these OSC inhibitors to ER positive human breast cancer cells (e.g. BT-474, T47-D, MCF-7) at a pharmacological dose or at a dose close to the IC_{50} value for OSC (nM range) reduced tumor cell viability in vitro. Administration of RO to animals with human breast cancer cell-derived xenografts prevented further in vivo progression of the disease, with no apparent toxicity. Since BT-474 cells are also tamoxifen resistant and rich in Her2/neu, RO appears to be effective in vivo against even the most aggressive anti-hormone resistant tumors. Importantly, RO had no effect on the viability of normal human mammary cells. Our study shows for the first time that inhibition of cholesterol biosynthesis using OSC inhibitors is a novel and potent means by which to destroy human breast cancer cells, though further studies are necessary to determine the mechanism of RO mediated loss of breast cancer cell viability. Supported by a Dept of Defense Breast Cancer Pgm grant W81XWH-12-1-0191, NIH grant R21 GM088517, and by a COR grant from the University of Missouri, Columbia.

Fig 1A: Ro 48-8071 inhibits viability of breast cancer cells in a dose- and time-dependent manner

SRB assay was used to measure cell viability

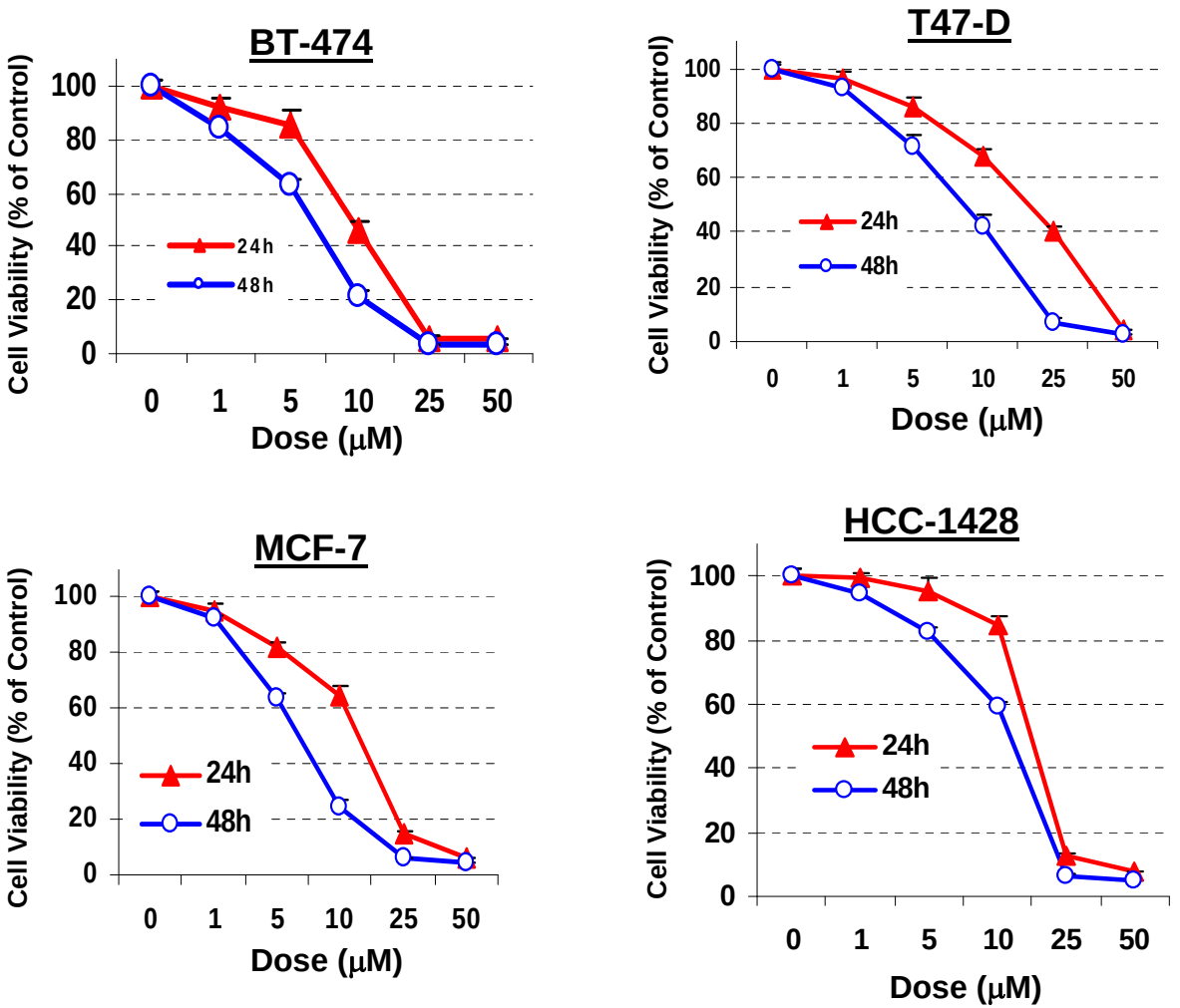


Fig 1 B: IC_{50} of Ro- 48-8071 on various breast cancer cells

IC_{50} of RO 48-8071 for various breast cancer cells

Cell lines	IC_{50} (μM) (24 hrs)	IC_{50} (μM) (48 hrs)
BT-474	9.514 ± 0.05	6.060 ± 0.23
T47-D	11.532 ± 0.36	7.760 ± 0.29
MCF-7	12.320 ± 0.59	6.335 ± 0.34
HCC-1428	14.644 ± 0.42	11.578 ± 0.34
ZR-75	11.038 ± 0.29	7.632 ± 0.30

Fig 1 C: Ro 61-3479 also has potent antitumor effect
(SRB assay was used)

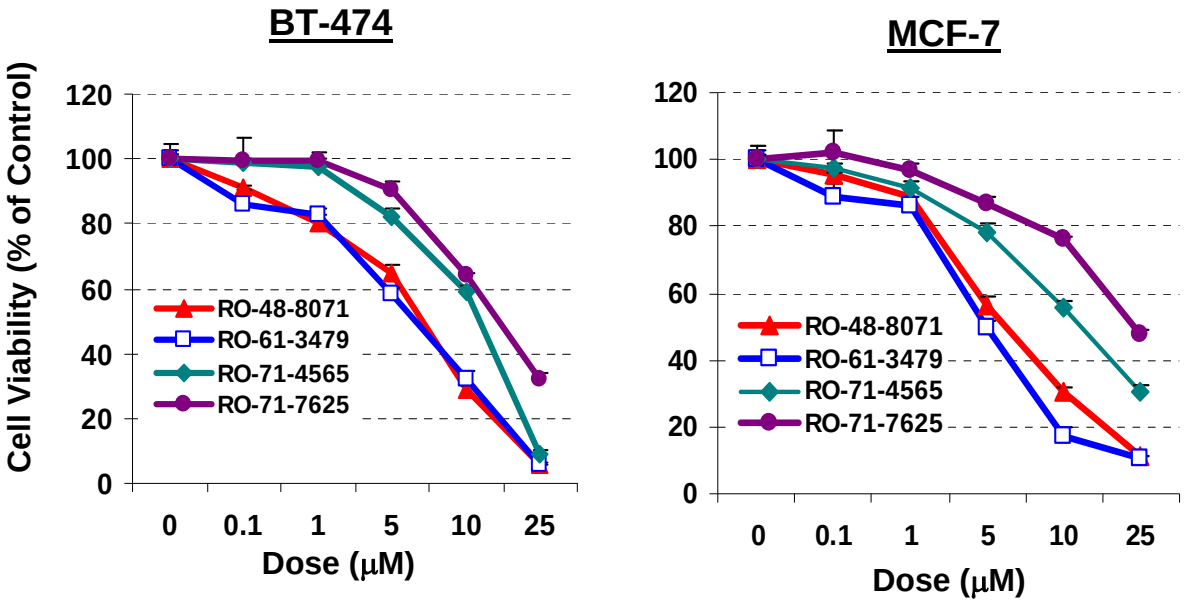


Fig 1D: Ro 48-8071 has minimal effect on growth of normal mammary cells

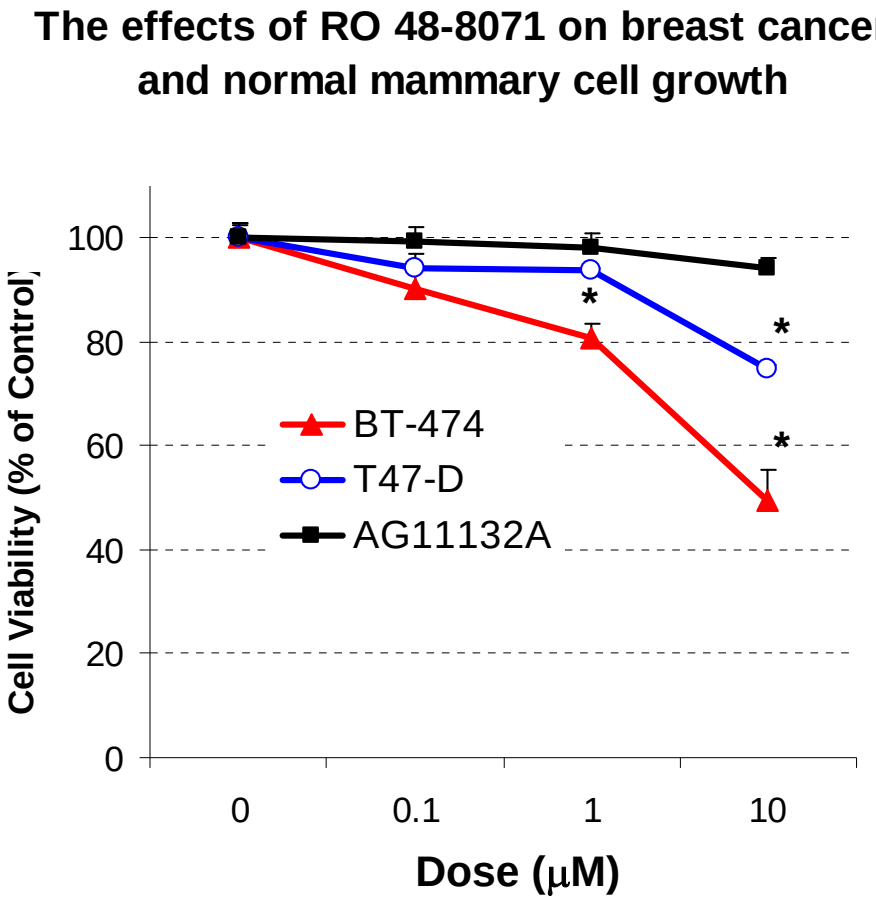


Fig 1E: Statins have reduced effect on growth of BT-474 breast cancer cell compared with Ro

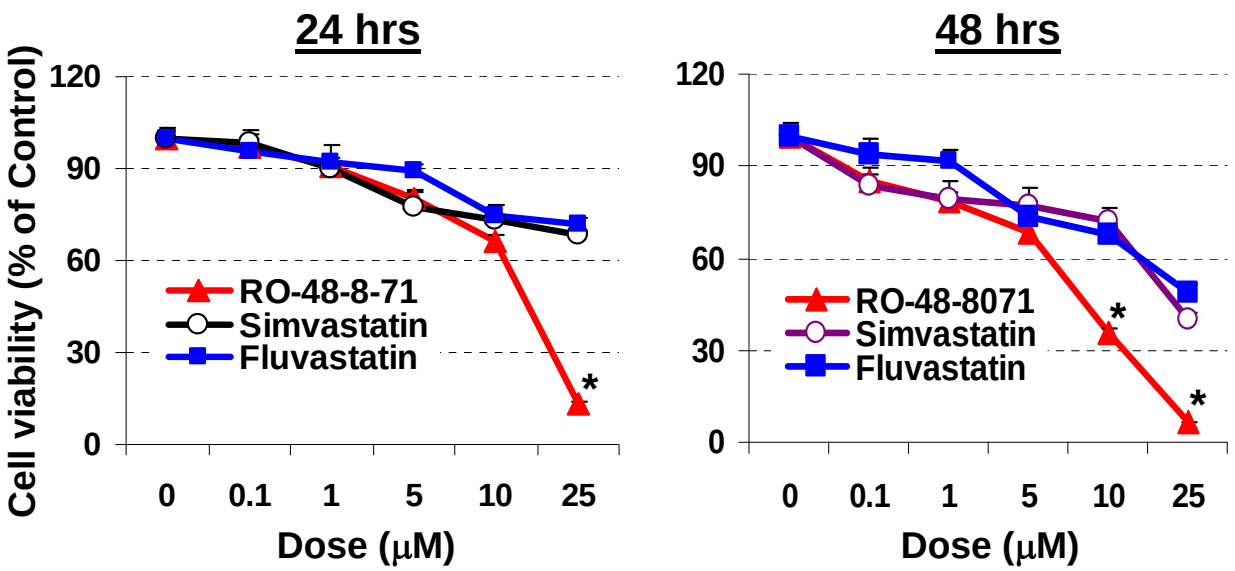


Fig 2A. Ro 48-8071 degrades ER-α and up-regulates ER-β in breast cancer cells

(upper panel, time in hours; lower panel, dose in μM)

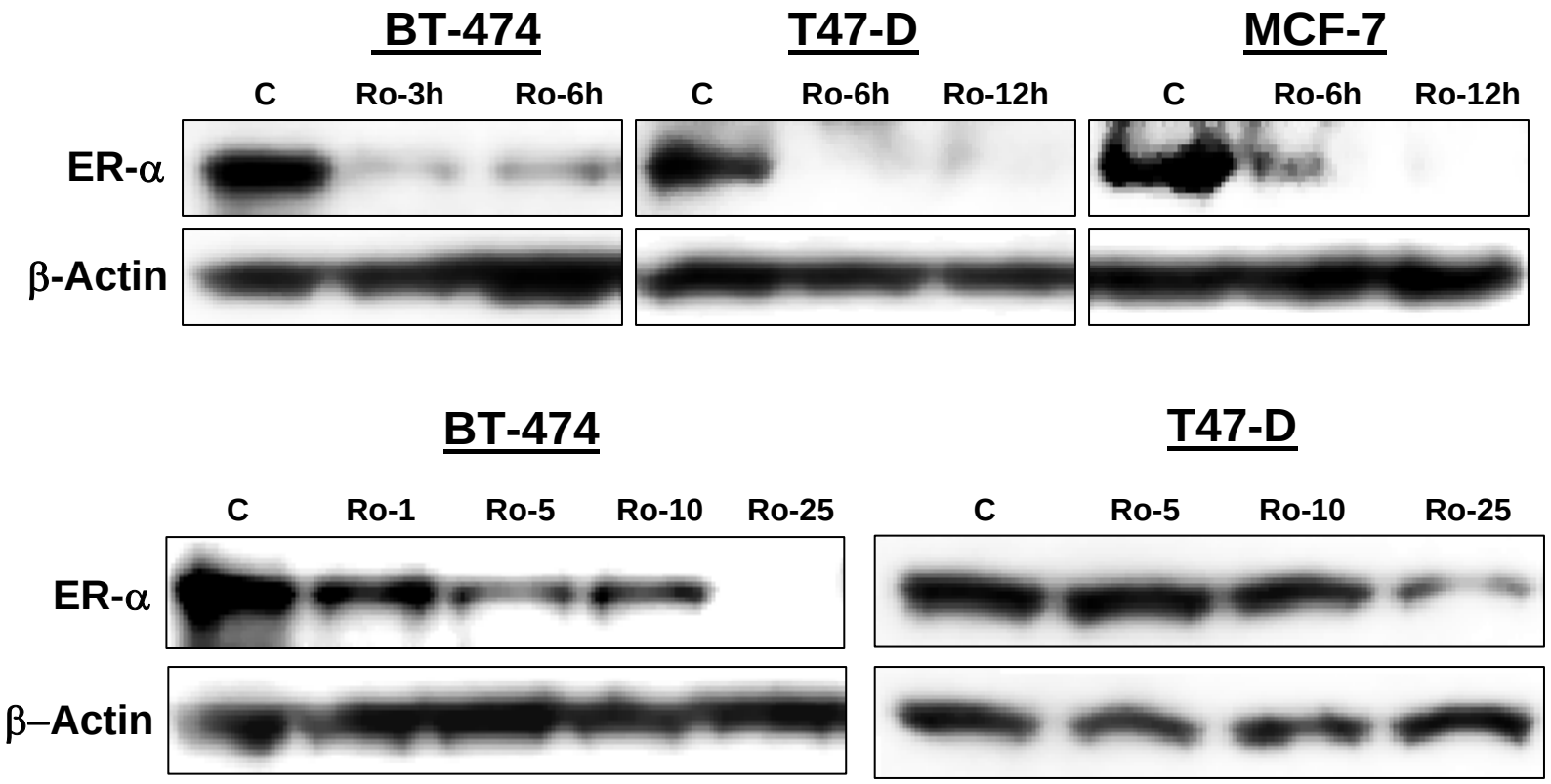


Fig 2B: Ro degrades ERa in a proteosomal-dependent manner

(M=MG132, proteosome inhibitor used in μM)

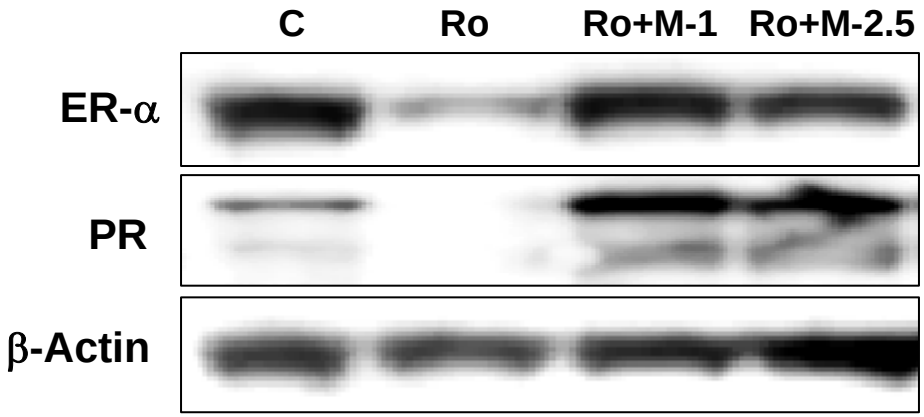


Fig 2C: Time and Dose-dependent up-regulation of ER-β expression by Ro 48-8071 (Western blot analysis)

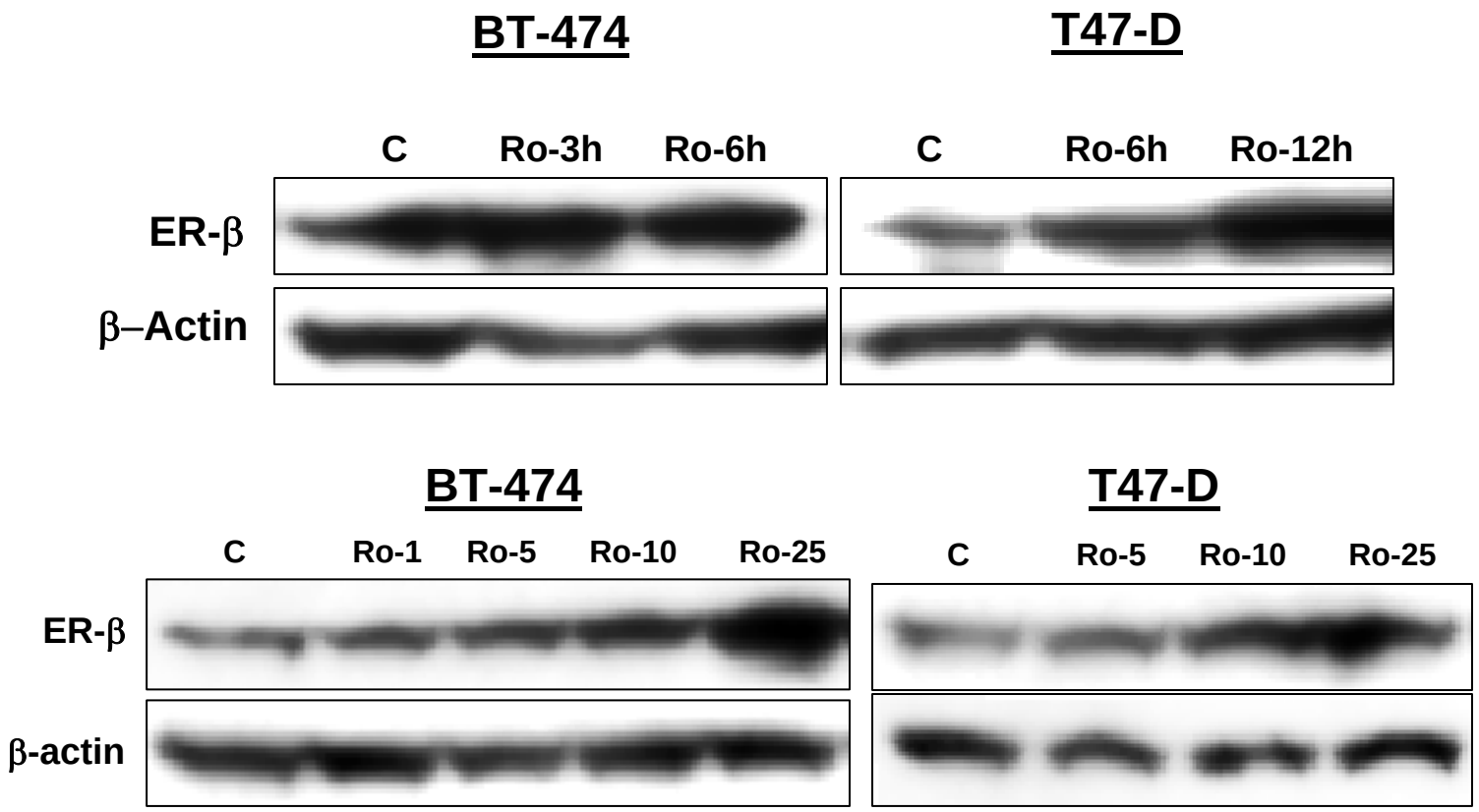


Fig 2D: Inhibition of ERα and up-regulation of ER-β expression by Low-dose (nM) Ro 48-8071 (Western blot analysis)

Fig 3: Inhibition of E2 induced cell proliferation by Ro 48-8071 (SRB Assay; RO in μM))

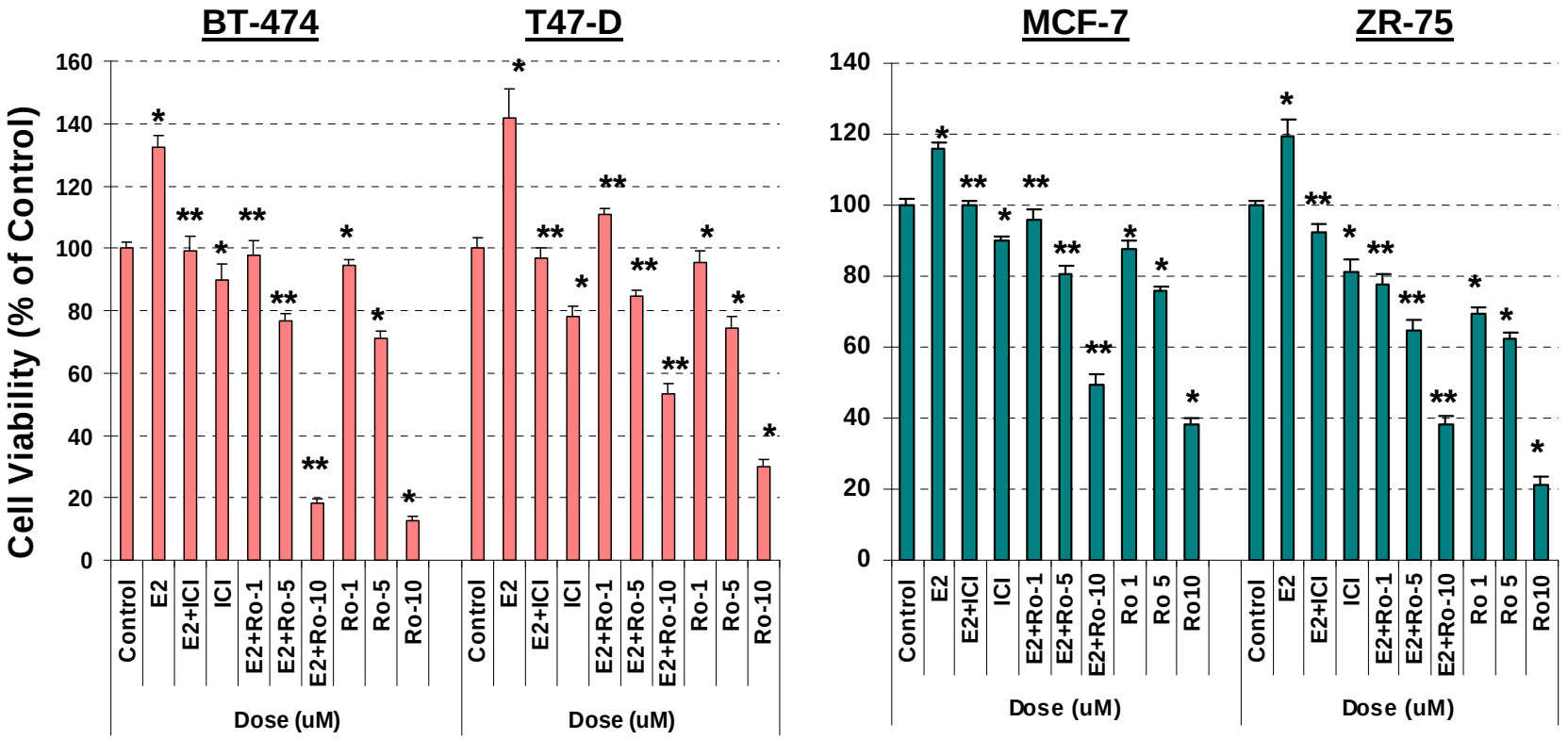
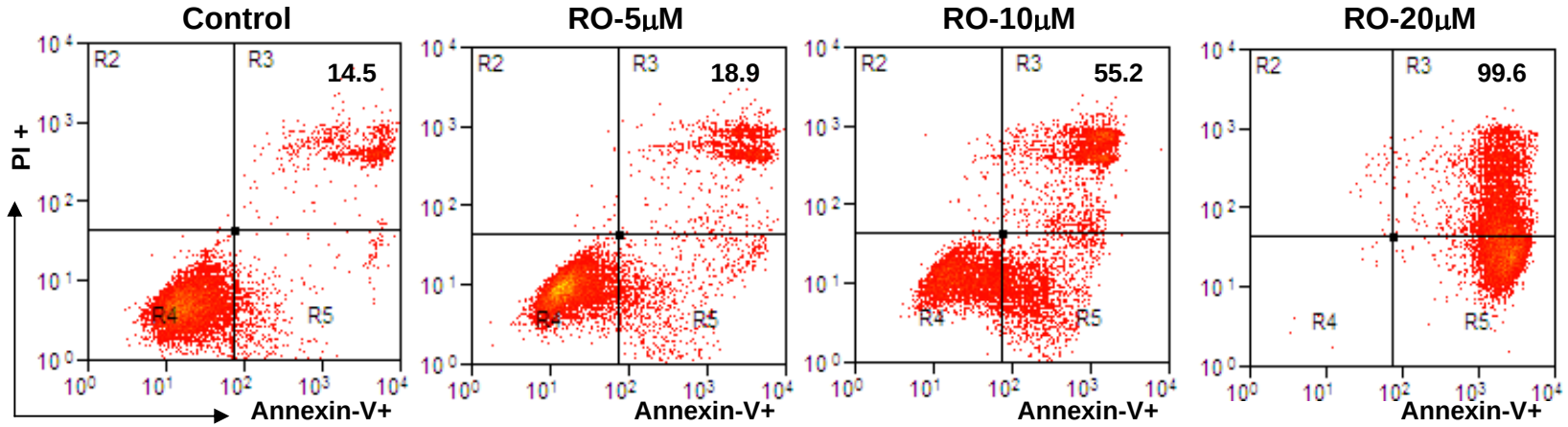


Fig 4A: Ro 48-8071-mediated induction of breast cancer cell death (FACS analysis)

BT-474



MCF-7

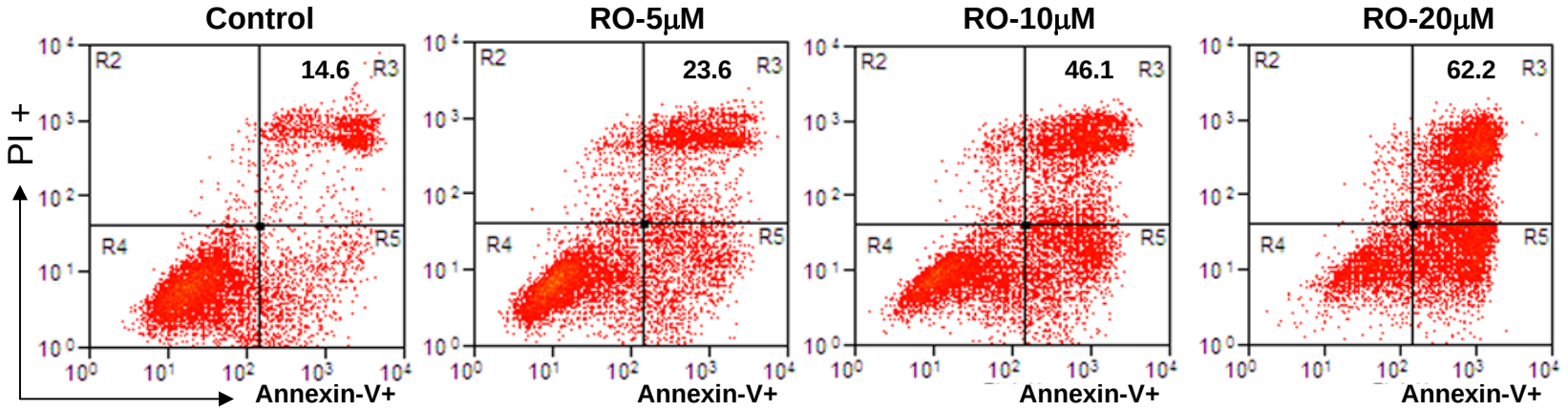


Fig 4B: Data Analysis from expt shown in Fig 4A run in triplicates

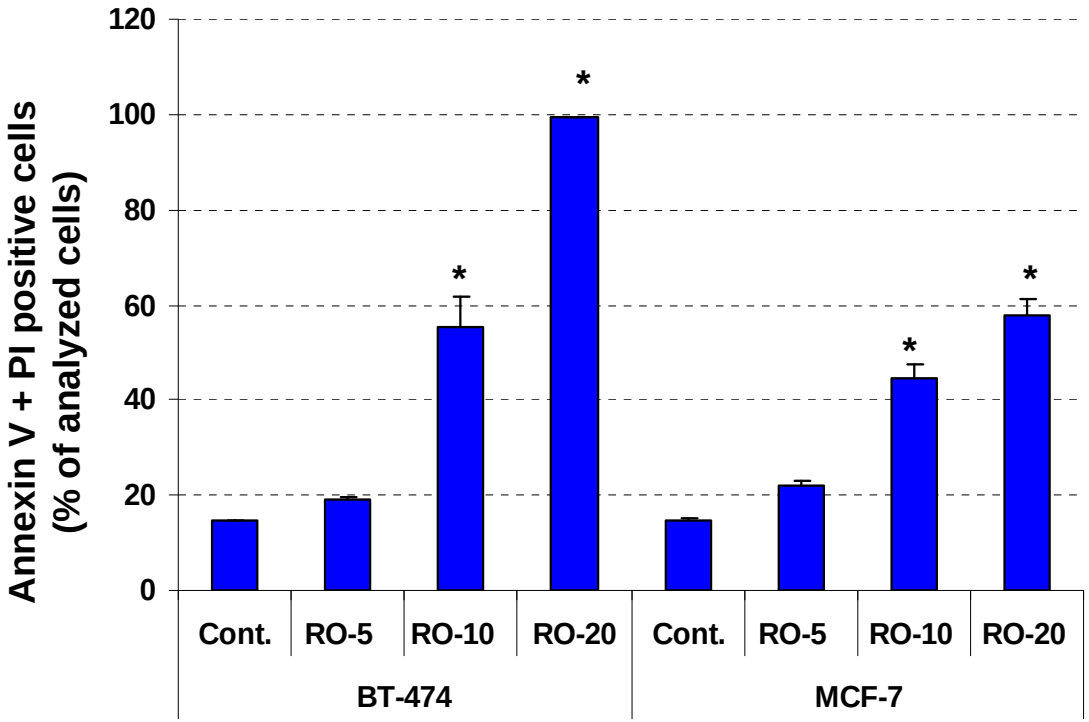
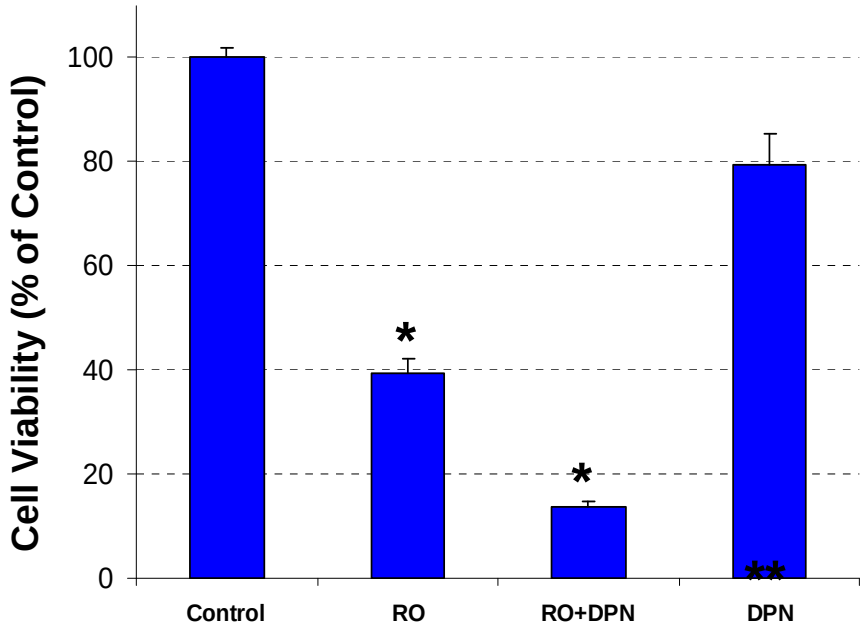


Fig 4C. An additive effect of Ro 48-8071 combined with DPN on inhibition of BT-474 cell growth



DPN is an ERβ specific ligand

Fig 5A: Induction of cell cycle arrest related protein p21 in breast cancer cells

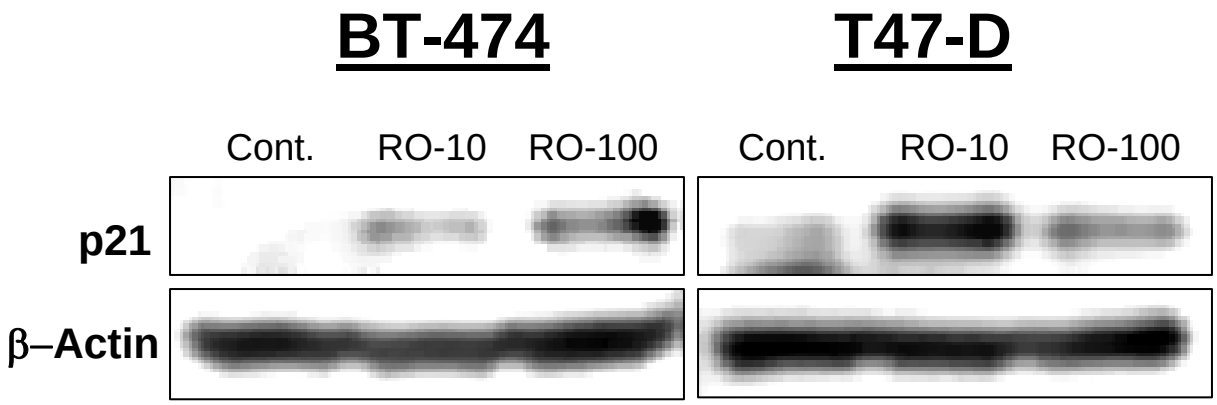


Fig 5B: Inhibition of breast cancer cell survival protein

Fig 6. Knockdown of ERβ abolished anti-proliferative effects of Ro 48-8071

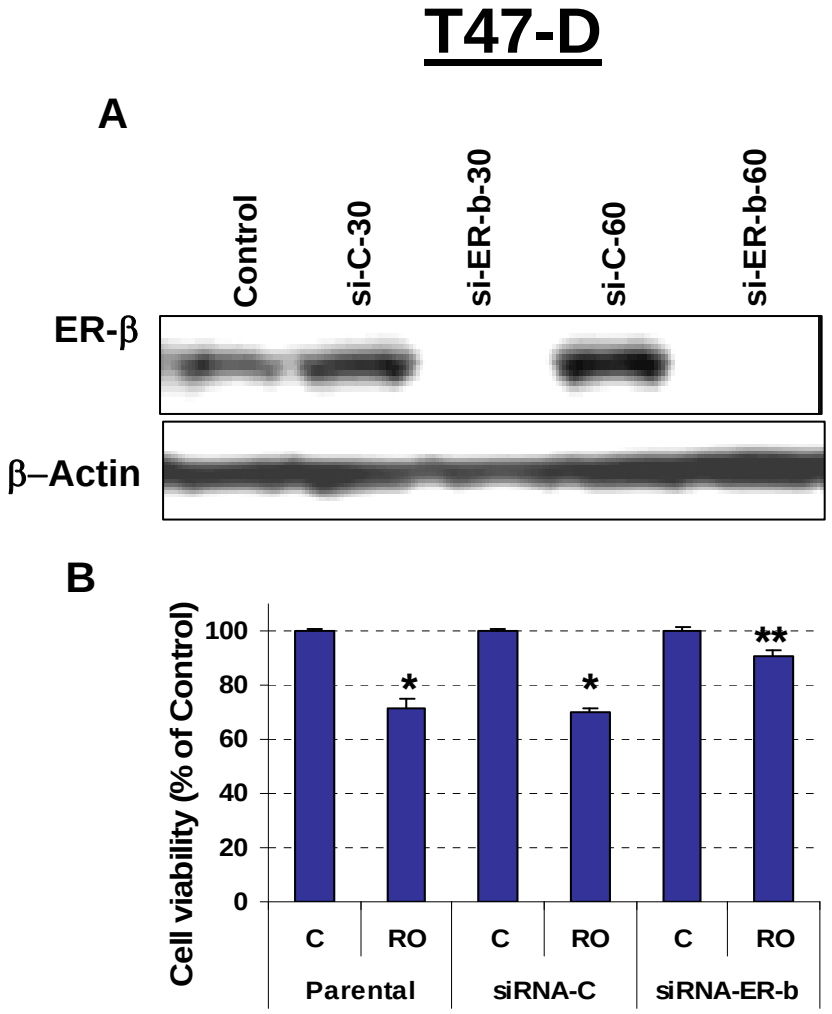


Fig 7A: Inhibition of tumor growth in vivo in nude mice

A:

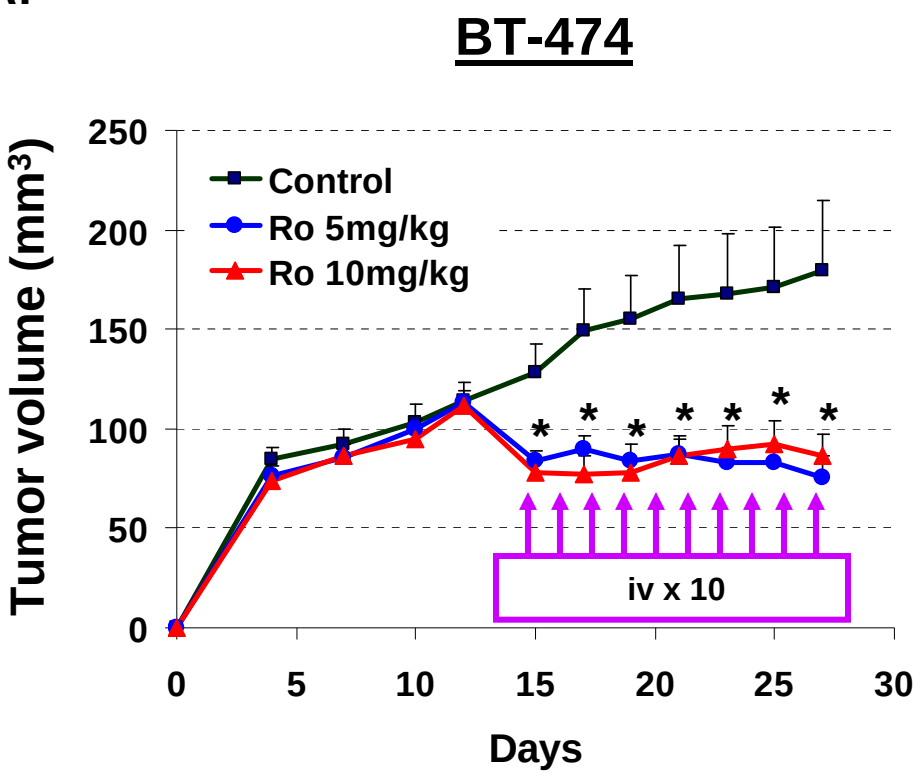


Fig 7B: No adverse treatment effect on animals during experiment shown in 7A